Tuberculosis 94 (2014) 374–382

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

IMMUNOLOGICAL ASPECTS

Differences in immune cell function between tuberculosis positive and negative Asian elephants

Jennifer A. Landolfi [a,*], Michele Miller [b], Carol Maddox [c], Federico Zuckermann [d], Jennifer N. Langan [e,f], Karen A. Terio [a]

[a] University of Illinois, Zoological Pathology Program, Loyola University Medical Center, Building 101, Room 0745, 2160 South First Avenue, Maywood, IL 60153, USA
[b] Rare Species Conservatory Foundation, 1222 E Road, Loxahatchee, FL 33470, USA
[c] University of Illinois, Department of Pathobiology, 1219 VMVSB, 2001 South Lincoln, Urbana, IL 61802, USA
[d] University of Illinois, Department of Pathobiology, 2834 VMBSC, 2001 South Lincoln, Urbana, IL 61802, USA
[e] University of Illinois, Department of Veterinary Clinical Medicine, 1008 West Hazelwood Drive, Urbana, IL 61802, USA
[f] Chicago Zoological Society, Brookfield Zoo, Brookfield, IL 60513, USA

ARTICLE INFO

Article history:
Received 10 October 2013
Received in revised form
23 January 2014
Accepted 1 March 2014

Keywords:
Elephant
Elephas maximus
Tuberculosis
Immunity
Cytokine

SUMMARY

Tuberculosis is an important health concern for Asian elephant (*Elephas maximus*) populations worldwide, however, mechanisms underlying susceptibility to *Mycobacterium tuberculosis* are unknown. Proliferative responses assessed via brominated uridine incorporation and cytokine expression measured by real-time RT-PCR were evaluated in peripheral blood mononuclear cell (PBMC) cultures from 8 tuberculosis negative and 8 positive Asian elephants. Cultures were stimulated with *Mycobacterium bovis* purified protein derivative (PPD-B), *M. tuberculosis* culture filtrate protein (CFP)-10, and *Mycobacterium avium* PPD (PPD-A). Following stimulation with PPD-B, proliferation was higher ($\alpha = 0.005$) in positive samples; no significant differences were detected following CFP-10 or PPD-A stimulation. Tumor necrosis factor (TNF)-α, interleukin (IL)-12, and interferon (IFN)-γ expression was greater in samples from positive elephants following stimulation with PPD-B ($\alpha = 0.025$) and CFP-10 ($\alpha = 0.025$ TNF-α and IL-12; $\alpha = 0.005$ IFN-γ). Stimulation with PPD-A also produced enhanced IL-12 expression in positive samples ($\alpha = 0.025$). Findings suggested that differences in immune cell function exist between tuberculosis positive and negative elephants. Proliferative responses and expression of TNF-α, IL-12, and IFN-γ in response to stimulation with PPD-B and CFP-10 differ between tuberculosis positive and negative elephants, suggesting these parameters may be important to tuberculosis immunopathogenesis in this species.

1. Introduction

Tuberculosis is an important health concern for elephant populations worldwide. The disease most commonly affects Asian elephants (*Elephas maximus*), and the majority of cases are due to *Mycobacterium tuberculosis*, the cause of human tuberculosis [1–4]. Since 1994, more than 50 culture confirmed cases of tuberculosis have been documented in U.S. captive elephants [2]. Recent studies have also reported confirmed cases and increased seroprevalance in populations of domesticated Asian elephants in range countries [5,6]. Elephant infections are typically chronic and subclinical; consequently diagnosis of infected individuals is challenging [1–3,7]. The current gold standard test, trunk wash mycobacterial culture, is insensitive [1,2,8,9]. Newer serologic tests have been developed and are recommended for screening. These tests have improved sensitivity and good specificity, however, validation is on-going [1,8–11]. In addition to individual animal and herd health concerns, elephant mycobacterial infection has significant public health implications. Throughout the world, captive elephants interact closely with human handlers for work and exhibition providing opportunity for exposure and potential zoonotic and/or anthropozoonotic transmission of disease [1,3,12,13]. The threat of disease transmission also extends to wild populations of elephants.

* Corresponding author. Tel.: +1 708 216 1185; fax: +1 708 216 5934.
E-mail addresses: landolfi@illinois.edu (J.A. Landolfi), mmiller@rarespecies.org (M. Miller), maddox@illinois.edu (C. Maddox), fazaaa@illinois.edu (F. Zuckermann), jennifer.langan@czs.org (J.N. Langan), kterio@illinois.edu (K.A. Terio).

http://dx.doi.org/10.1016/j.tube.2014.03.001
1472-9792/

Though infection has not been documented in wild elephants to date, captive working elephants in range countries frequently mingle with free-ranging elephants providing opportunity for disease transmission [1]. Public health vigilance and effective conservation of this endangered species require a better understanding of tuberculosis pathogenesis.

Unfortunately, the mechanisms underlying tuberculosis susceptibility in elephants are unknown, and information regarding elephant immune function is scarce [14–17]. In humans and other studied species, disturbances in the normal balance between cell-mediated (T_H1) and humoral (T_H2) immune responses are central to tuberculosis pathogenesis [18–28]. Evaluation of systemic immune responses in humans via measurement of cytokines has shown that inadequate T_H1 responses are a feature of active disease [19,27,29–34]. Considering altered immune responses to tuberculosis are instrumental in determining disease susceptibility and influencing pathogenesis in humans, it is plausible that immune function alterations may similarly contribute to Asian elephant tuberculosis susceptibility.

In a previous study, mRNA levels of several T_H1 and T_H2 cytokines significant in the pathogenesis of human tuberculosis were measured in peripheral whole blood samples from 106 (15% tuberculosis seropositive) Asian elephants using elephant-specific, real time RT-PCR assays [15]. Cytokine levels were measured in the absence of any mitogenic or antigenic stimulation. Though significant differences in levels of examined cytokines were not detected, the data illustrated some trends in cytokine expression between the two groups that warranted further investigation. Consequently, the current study was undertaken to measure proliferative responses and cytokine mRNA expression in peripheral blood mononuclear cell (PBMC) cultures from 8 tuberculosis negative and 8 tuberculosis positive North America-based Asian elephants following stimulation with mycobacterial antigens.

2. Materials and methods

2.1. Animals/sample collection

Samples were collected from 16 captive, North America-based, Asian elephants. For the purposes of this study, positive cases were designated as elephants with *M. tuberculosis* positive trunk wash cultures and/or seroreactivity to both the Elephant TB STAT PAK® screening test (Chembio, Medford, NY) and multiple antigen print immunoassay (MAPIA). Negative cases were those with no history of positive trunk wash culture or serology and no history of exposure to a known positive elephant. All study animals were zoo or retired performance elephants accustomed to human handlers and trained to cooperate voluntarily with routine veterinary examinations and procedures. Using minimal manual restraint, a total of 20 ml of peripheral whole blood was collected from the ear vein of each animal into collection tubes containing heparin sulfate (BD Biosciences, San Jose, CA). All samples were received for processing at the laboratory within 24 h of collection. In addition to blood samples, information regarding age, sex, current medical treatments and pertinent chronic inflammatory conditions was obtained for each of the study animals. Prior to experiments utilizing samples from the 16 study animals, preliminary experiments were conducted using samples from 2, tuberculosis negative Asian elephants to determine optimal parameters for culture of elephant PBMCs. Variables evaluated included: cell culture medium composition, concentration of viable PBMCs/ml, mitogen and antigen concentration for stimulation and incubation times following mitogen and antigen exposure [35]. Results of these preliminary experiments were used to determine the optimal parameters for culture and stimulation of elephant PBMCs utilized in the current study.

2.2. Isolation of PBMCs

Upon receipt, whole blood smears were examined via light microscopy to determine the relative proportions of monocytes and lymphocytes in each sample. Peripheral blood mononuclear cells were isolated from whole blood samples using density gradient centrifugation. From each sample, 15 ml of room temperature heparinized whole blood were diluted with 33 ml of room temperature Hank's Balanced Salt Solution (HBSS; Fisher Scientific, Pittsburgh, PA). The diluted sample was divided into six equal 8 ml aliquots, and each aliquot was carefully layered over 4 ml of room temperature Ficoll-Paque™ Plus, density 1.077 ± 0.001 g/ml (GE Healthcare, Uppsala, Sweden) in 15 ml sterile, plastic conical vials. Separation was achieved through centrifugation at 400 RCF for 30 min. Following centrifugation, the superficial buffer and plasma layers were removed from each vial via aspiration without disturbing the mononuclear cell layer interface. Next, the mononuclear cell layer was removed via aspiration and transferred to a new 15 ml vial. Mononuclear cells were washed with HBSS; individual aliquots from each sample were recombined and suspended in 37 °C cell culture medium. Cell culture medium consisted of RPMI 1640 medium with HEPES and L-glutamine (HyClone; Thermo Scientific, Waltham, MA) supplemented with 10% heat inactivated fetal calf serum (MP Biomedicals, Solon, OH), 1% 100 mM sodium pyruvate (Invitrogen, Carlsbad, CA), 1%, 10 mM minimal essential medium (Sigma–Aldrich, St. Louis, MO), 1% 5000 U penicillin/5000 Fg/ml streptomycin (Invitrogen), 0.1% 10 mg/ml gentamycin sulfate (Invitrogen) and 3.66×10^{-4}% beta mercaptoethanol (2×10^{-5} M; Fisher Scientific). Mononuclear cell viability was assessed directly in a 100 μl aliquot of each sample through evaluation of trypan blue dye (Sigma–Aldrich) retention (viable cells are able to pump dye out excluding the stain; nonviable cells retain stain). Each sample was then diluted with cell culture medium to a final concentration of 1×10^6 viable cells/ml.

2.3. Proliferation assays

For each sample, 100 μl aliquots (1×10^5 viable cells), in triplicate, were plated onto 96 well, clear plastic, flat bottom culture plates (Falcon® 3872 Primaria®; Becton Dickinson, Lincoln Park, NJ). Samples were incubated for 5 days at 37 °C, 5% CO_2 in the presence of 20 μg/ml *Mycobacterium bovis* purified protein derivative (PPD-B; Prionics, Zurich, Switzerland), 20 μg/ml *M. tuberculosis* culture filtrate protein (CFP)-10 (BEI Resources, Manassas, VA), and 20 μg/ml *Mycobacterium avium* PPD (PPD-A; Prionics). Unstimulated sample aliquots and cell-free medium controls were included to serve as negative controls. Aliquots cultured in the presence of 6 μg/ml concanavalin A (ConA; Sigma–Aldrich) were included as positive controls. Following the 5-day incubation, proliferating cells were distinguished and quantified via brominated uridine (BrdU) incorporation using a commercially available cell proliferation 5-bromo-2′deoxyuridine ELISA kit (Roche Diagnostics Corporation; Indianapolis, IN) according to manufacturer's instructions. Colorimetric reactions were assessed via spectrophotometry at 20 min post addition of kit substrate (SPECTRAmax PLUS; Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmaxPRO software (Version 3.1.1; Molecular Devices). Absorbance values obtained via spectrophotometry for each sample triplicate were averaged to produce a single representative value. Any sample in which triplicate values varied more than one standard deviation from the mean were rejected and reanalyzed. Then, absorbance values for stimulated sample aliquots were divided by absorbance

values for the unstimulated sample aliquots to determine the stimulation index for each sample. Differences in stimulation indices between positive and negative samples were evaluated for statistical significance with Mann–Whitney *U* tests.

2.4. Quantification of cytokine expression

2.4.1. Culture and RNA extraction

For each sample, 100 μl aliquots (1×10^5 viable cells), in triplicate, were plated onto a second 96 well, clear plastic, flat bottom culture plate for quantification of cytokine mRNA levels. Samples were incubated for 2 days at 37 °C, 5% CO_2 in the presence of 20 μg/ml PPD-B (Prionics), 20 μg/ml CFP-10 (BEI Resources), and 20 μg/ml PPD-A (Prionics). Negative and positive controls were also included as described for proliferation assays above. Following incubation, each culture triplicate was combined and transferred to a microcentrifuge tube. Samples were pelleted at 18,000 RCF for 5 min. Supernatant was discarded and RNA was extracted from cell pellets using a commercially available kit (RiboPure; Ambion, Austin, TX) according to manufacturer's instructions. Concentration of extracted RNA was measured using a NanoDrop™ 2000 spectrophotometer (Fisher Scientific). To synthesize cDNA for use in real time PCR assays, 10 ng of RNA were reverse transcribed per sample in a 90-μl reaction using a commercially available kit (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA).

2.4.2. Real time RT-PCR assays

Quantitative, real time RT-PCR assays previously developed and validated for specific use in elephant samples were utilized to measure mRNA levels of tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β [16]. Levels of interferon (IFN)-γ and interleukin (IL)-12 were measured using newly developed and validated, elephant-specific assays with increased sensitivity (Table 1). All real time PCR reactions were run in a simplex format in triplicate and contained Taqman® Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 250 nmol probe, 900 nmol of both forward and reverse primers, and RNAse-free water to a final reaction volume of 25 μl. Reactions also included a commercially available exogenous, non-template, internal control (Taqman® Exogenous Internal Control; Applied Biosystems) to confirm PCR amplification and detect potential amplification inhibitors within samples. Real time RT-PCR assays were performed as previously described [15,16]. In addition to negative controls, each plate included cytokine-specific standards (diluted elephant PCR product, $1{:}1 \times 10^6$ and $1{:}1 \times 10^8$) used to monitor and control for interassay variation. Amplification products of sample replicates were evaluated for consistency; variation among each sample's replicate threshold amplification (Ct) values used in later quantitative calculations was less than one standard deviation from the mean.

2.4.3. Housekeeping gene selection

To normalize the real time RT-PCR data for quantitative calculations, an appropriate housekeeping gene (HKG) unaffected by the experimental conditions (culture stimulation) was required. In order to assess candidate HKGs, levels of 18S rRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta actin were measured in both unstimulated and stimulated aliquots in a subset of the samples ($N = 6$; tuberculosis positive = 4, tuberculosis negative = 2). Levels of GAPDH and beta actin were measured using previously developed and validated elephant specific assays [16]. Levels of 18S rRNA were measured using a commercially available assay (Taqman® Pre-Developed Assay Reagents – Human 18S rRNA; Applied Biosystems) according to manufacturers' guidelines. Differences in Ct values between the unstimulated and stimulated aliquots for each sample were calculated for each candidate HKG. The HKG with the lowest differences between unstimulated and stimulated aliquots represented the candidate least influenced by culture stimulation; this HKG was then used for subsequent normalization of the elephant cytokine real time RT-PCR data.

2.4.4. Data analysis

Relative quantification of cytokine mRNA amplification was determined using the Pfaffl Method [36]. Replicate threshold amplification (Ct) values for each sample were averaged. For each sample, values obtained from unstimulated cultures served as calibration controls. In cases where the Pfaffl method could not be employed to calculate fold difference in mRNA levels due to lack of cytokine amplification in the unstimulated sample aliquot, normalized cytokine Ct values (cytokine average Ct – HKG average Ct) were examined instead. Differences in calculated values between positive and negative samples were evaluated for statistical significance with Mann–Whitney *U* tests.

3. Results

3.1. Animal demographic information and tuberculosis status

Study animal demographic information and tuberculosis status are summarized in Table 2. The mean age for the positive animals was 41 years (range 29–60); the mean age for the negative animals was 34.5 years (12–45). All elephants were female with exception of a single male (bull) elephant in the positive group. Based on the most recent annual test results, four of the tuberculosis positive animals were *M. tuberculosis* culture positive; the remaining four were seropositive on both tests but culture negative at the time of sample collection. Intervals between the last positive trunk wash culture and/or serologic testing and sampling for PBMC culture are presented in Table 2. At the time of sampling, Animal 3 had been under treatment (isoniazid and pyrazinamide) for approximately 1 year. Animal 2 had initiated treatment (rifampin and enrofloxacin) approximately 2 months prior to sampling. The remaining six positive animals had not received any anti-tuberculosis medications. All negative animals were culture negative, non-reactive on the most recent Elephant TB STAT-PAK®, and lacked history of exposure to any known positive elephants. The MAPIA test was not done in the negative elephants, because this assay is used as a

Table 1
Newly developed, Asian elephant-specific interferon (IFN)-γ and interleukin (IL)-12 real time RT-PCR assays.

	Primers*	Probe	Efficiency†	Sensitivity§
IL-12	**F**: ATGCAAAGCTTTTGATGGACC **R**: AAATTCAGGGCCTGCAT	ACATGTTGGCAGCTAT	96%	34.4
IFN-γ	**F**: GGAATATCTTAATGCAACTGATTCA **R**: CCTGGTTGTCTTTCAAGTTGTCAA	GAAGAACTGGAAAGAGGAG	97%	3

* All primer/probe sequences are listed 5′ to 3′. **F** = forward primer; **R** = reverse primer.
† Efficiency is calculated as $10^{(-1/\text{slope})} - 1$.
§ Sensitivity represents the minimal detectable number of dsDNA copies within 10 μl of sample (copy number = [ng DNA * 6.022×10^{23}]/[length (bps) * 1×10^9 ng/g * 650 g/mole of bps]). DNA concentration was determined via spectrophotometry.

Table 2
Asian elephant demographic information and tuberculosis status.

Sample	Sex	Age (years)	Culture*	STAT-PAK†	MAPIA§	Testing interval (months)**	Concurrent conditions
1	F	37	+	+	+	3	SA‡
2	F	40	+	+	+	4	None
3	F	60	+	+	+	<1	OA¶; SA
4	F	41	+	–	Inconclusive	12	None
5	M	29	–	+	+	4	OA; SA
6	F	35	–	+	+	<1	OA; SA
7	F	44	–	+	+	6	OA; SA
8	F	44	–	+	+	4	OA
9	F	12	–	–	NA	6	None
10	F	22	–	–	NA	6	None
11	F	38	–	–	NA	6	None
12	F	38	–	–	NA	7	OA
13	F	40	–	–	NA	8	OA; SA
14	F	40	–	–	NA	8	OA; SA
15	F	41	–	–	NA	6	None
16	F	45	–	–	NA	7	OA; SA

* Mycobacterial culture of trunk wash sample.
† Elephant TB STAT-PAK® serologic test.
§ Multiple antigen print immunoassay.
** Time in months between last positive trunk wash culture and/or serologic testing and sampling for PBMC culture.
‡ SA = pedal sole abscess.
¶ OA = osteoarthritis.

confirmatory test for animals that have Elephant TB STAT-PAK® positive results. In addition to demographics and tuberculosis status, general information regarding the presence of common chronic inflammatory conditions was obtained for each of the study animals. In six of the positive animals and four of the negative animals, chronic inflammatory conditions (osteoarthritis and/or pedal sole abscesses, specifically) were reported. In all cases, these conditions were considered clinically mild.

3.2. Isolation of PBMCs

Elephant PBMCs isolated via density gradient centrifugation had greater than 98% viability for all samples. Cytologically, smears from all samples had similar proportions of monocytes and lymphocytes. The average lymphocyte to monocyte ratio for the negative samples was 1.1999 ± 0.253 and was 1.2391 ± 0.324 for the positive samples. No significant difference was detected in the lymphocyte to monocyte ratio between the two groups using a student's t test ($p = 0.789$).

3.3. Proliferation assays

Proliferation, as quantified by BrdU incorporation, was present in ConA stimulated aliquots for all samples confirming viability in culture (Figure 1). Trends towards greater proliferation were noted in the positive group for all stimulants (Figure 1). Significant differences in stimulation indices between the positive and negative groups as indicated by Mann–Whitney U test results were present for ConA ($\alpha = 0.025$) and PPD-B ($\alpha = 0.005$).

3.4. Quantification of cytokine expression

For all assays, amplification of exogenous internal positive control DNA confirmed PCR amplification. Diluted elephant PCR product standards amplified as expected for all cytokines ensuring consistent assay efficiency and minimal interassay variation. Levels of IL-12, TNF-α, and TGF-β were measurable in unstimulated and stimulated culture aliquots for all samples. Interferon-γ was below the level of detection in unstimulated culture aliquots for the majority (13/16) of samples.

To facilitate selection of an appropriate housekeeping gene for normalization of real time PCR data, levels of GAPDH, beta actin, and 18S rRNA were measured in the subset of 6 samples. No statistically significant differences were detected between the 3 evaluated HKGs using paired t tests. Examination of raw data did show that 18S rRNA had the lowest average differences between unstimulated and stimulated aliquots for all stimulants (Table 3). These findings suggested 18S rRNA was the HKG least influenced by culture stimulation, and thus it was consequently utilized for normalization of the elephant cytokine real time RT-PCR data.

For all samples and all cytokines, ConA stimulation resulted in increased cytokine levels as compared with levels in unstimulated aliquots, confirming the use of this mitogen as a positive control for stimulation of cytokine mRNA expression in elephant PBMC cultures. Differences in cytokine fold difference values (TNF-α, IL-12, TGF-β) between the positive and negative groups following ConA stimulation, however, were not statistically significant. Following PPD-B stimulation, fold difference values for both TNF-α and IL-12 were higher in the positive group, and differences between the positive and negative groups were statistically significant ($\alpha = 0.025$). Similar results were seen for TNF-α and IL-12 with CFP-

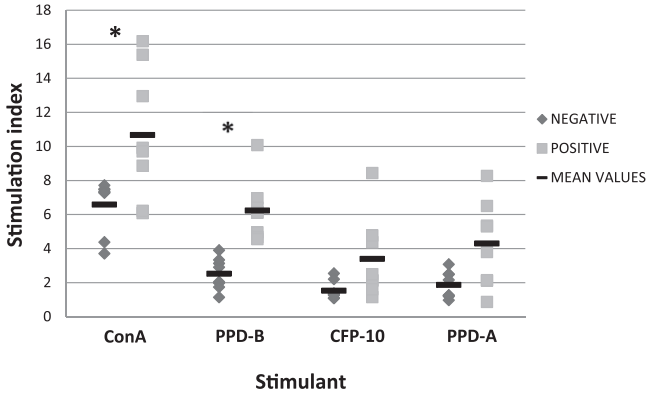

Figure 1. Graph depicts proliferative response as measured by stimulation index of Asian elephant PBMCs following stimulation in culture. Positive samples were from animals with positive trunk wash mycobacterial culture and/or seropositivity with both the Elephant TB STAT-PAK® and MAPIA tests. ConA = concanavalin A; PPD-B = *Mycobacterium bovis* purified protein derivative; CFP-10 = *M. tuberculosis* culture filtrate protein 10; PPD-A = *M. avium* purified protein derivative. Asterisks denote stimulants for which stimulation indices were significantly different between positive and negative groups (ConA $\alpha = 0.025$; PPD-B $\alpha = 0.005$).

Table 3
Determination of appropriate housekeeping gene for normalization of Asian elephant PBMC culture real time PCR data.

HKG*	$Ct^{\dagger}_{Unstimulated} - Ct_{ConA}$	$Ct_{Unstimulated} - Ct_{PPD\text{-}B}$	$Ct_{Unstimulated} - Ct_{CFP\text{-}10}$	$Ct_{Unstimulated} - Ct_{PPD\text{-}A}$
GAPDH§	2.24	1.69	1.70	1.50
Beta actin	2.30	1.81	1.34	1.77
18S rRNA	1.60	1.13	1.28	1.17

* Housekeeping gene.
† Threshold amplification value as determined by real time RT-PCR.
§ Glyceraldehyde 3-phosphate dehydrogenase.

10 stimulation ($\alpha = 0.025$; Figures 2 and 3). No statistically significant difference in TNF-α levels between the two groups was detected following PPD-A stimulation. In the case of IL-12, however, PPD-A stimulation did result in a significant difference between the two groups ($\alpha = 0.025$). Significant differences in TGF-β levels between the positive and negative groups were not present with any of the tested mycobacterial antigens.

For the IFN-γ data, lack of amplification in the majority of unstimulated sample aliquots precluded conventional calculation of fold difference using the Pfaffl method. Alternatively, normalized cytokine Ct values (cytokine average Ct − HKG average Ct) were determined for each of the culture stimulants and compared between the two groups. Following ConA, PPD-B and CFP-10 stimulation, IFN-γ normalized Ct values were, on average, lower in the positive group (lower normalized Ct values correlate with higher mRNA levels within samples), and differences between the positive and negative groups were statistically significant (ConA and PPD-B, $\alpha = 0.025$; CFP-10, $\alpha = 0.005$; Figure 4). No statistically significant difference in IFN-γ normalized Ct values following PPD-A stimulation was detected between the two groups.

Comparison of positive elephants that were culture positive vs. seropositive on both the Elephant TB STAT-PAK® and MAPIA demonstrated additional stratification of the above findings. Specifically, following both PPD-B and CFP-10 stimulation, highest IL-12 fold difference values were noted for the culture positive samples with values for the seropositive samples intermediate between culture positive and negative samples (Figure 5(a)). For IFN-γ, similar trends were apparent with seropositive samples exhibiting intermediate values (Figure 5(b)). These results were not analyzed statistically due to diminished sample size created by separation of the positive group into culture positive and seropositive subgroups.

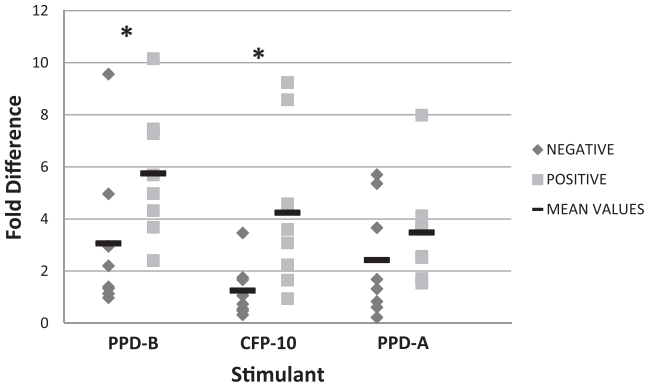

Figure 2. Graph depicts tumor necrosis factor-α levels as measured by mRNA fold difference of Asian elephant PBMCs following stimulation in culture. Positive samples were from animals with positive trunk wash mycobacterial culture and/or seropositivity with both the Elephant TB STAT-PAK® and MAPIA tests. PPD-B = *Mycobacterium bovis* purified protein derivative; CFP-10 = *M. tuberculosis* culture filtrate protein 10; PPD-A = *M. avium* purified protein derivative. Asterisks denote stimulants for which fold difference values were significantly different between positive and negative groups ($\alpha = 0.025$).

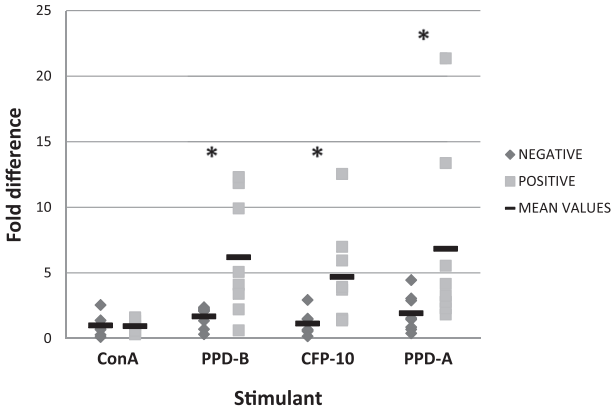

Figure 3. Graph depicts interleukin-12 levels as measured by mRNA fold difference of Asian elephant PBMCs following stimulation in culture. Positive samples were from animals with positive trunk wash mycobacterial culture and/or seropositivity with both the Elephant TB STAT-PAK® and MAPIA tests. ConA = concanavalin A; PPD-B = *Mycobacterium bovis* purified protein derivative; CFP-10 = *M. tuberculosis* culture filtrate protein 10; PPD-A = *M. avium* purified protein derivative. Asterisks denote stimulants for which fold difference values were significantly different between positive and negative groups ($\alpha = 0.025$).

4. Discussion

The findings of this study suggest that differences in immune cell function do exist between tuberculosis positive and negative elephants. Though additional studies with larger numbers of samples would be ideal for confirmation, results presented here provide evidence for host immune system contribution in the pathogenesis of elephant tuberculosis. Specifically, proliferative responses and expression of TNF-α, IL-12, and possibly IFN-γ in response to stimulation with PPD-B and CFP-10 were, on the whole, different between tuberculosis positive and negative individuals. Differences in measured immune responses between the two groups could have been direct manifestations of infection. Alternatively, noted differences may represent functional aberrations in the positive group that could have contributed to susceptibility. In either case, results suggested these parameters may influence tuberculosis immunopathogenesis in this species, and in future studies, could prove to be useful diagnostic markers.

The antigens, PPD-B, CFP-10, and PPD-A, utilized in this study were all protein antigens. Accordingly, generation of measured responses in the elephant PBMC samples required uptake, processing and presentation of antigens by antigen presenting cells (monocytes/macrophages) followed by subsequent recognition by antigen specific T lymphocytes (both of which were components of the elephant PBMC cultures). The positive control, ConA, a nonspecific and direct T lymphocyte mitogen, provided for antigen-independent stimulation to confirm sample viability. Activation of T lymphocytes afforded directly by ConA stimulation or as a result of antigen recognition by specific T lymphocyte subsets led to proliferation mediated in an autocrine fashion primarily by IL-2. Activated T lymphocytes were also the source of IFN-γ expression. Expression of IL-12 originated from activated monocytes/macrophages. Monocyte/macrophage activation could have been facilitated by innate mechanisms (i.e. direct antigen contact and phagocytosis) and/or adaptive, cell-mediated mechanisms involving T lymphocyte elaboration of IFN-γ. Both activated T lymphocytes and monocytes/macrophages in study samples could have been sources for TNF-α and TGF-β expression [21,24,37]. To function as a "broad spectrum" antigen and ensure robust responses by the elephant PBMCs, this study employed PPD-B, a complex antigen containing multiple *M. tuberculosis* complex

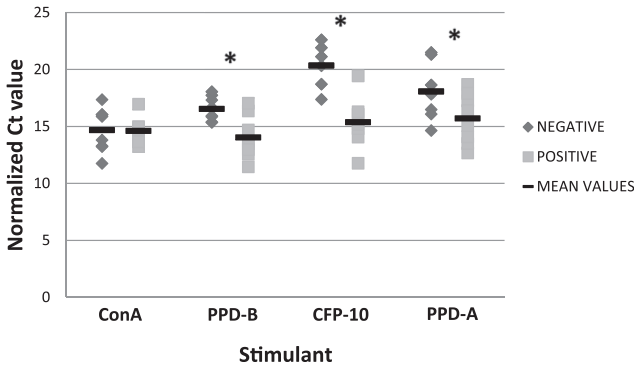

Figure 4. Graph depicts IFN-γ levels, represented by normalized average Ct values, of Asian elephant PBMCs following stimulation in culture. Positive samples were from animals with positive trunk wash mycobacterial culture and/or seropositivity with both the Elephant TB STAT-PAK® and MAPIA tests. ConA = concanavalin A; PPD-B = *Mycobacterium bovis* purified protein derivative; CFP-10 = *M. tuberculosis* culture filtrate protein 10; PPD-A = *M. avium* purified protein derivative. Asterisks denote stimulants for which normalized Ct values were significantly different between positive and negative groups (ConA and PPD-B $\alpha = 0.025$; CFP-10 $\alpha = 0.005$).

derived proteins, for stimulation in addition to CFP-10. Though culture of positive elephants yielded *M. tuberculosis* (and not *M. bovis*), genomic similarity between *M. bovis* and *M. tuberculosis* and homology among protein antigens of all *M. tuberculosis* complex organisms indicated that stimulation with PPD derived from either organism would be expected to produce comparable effects [38–40].

Both PPD-B and CFP-10 stimulation resulted in higher levels of TNF-α mRNA in positive samples, and differences in levels between the positive and negative groups were statistically significant

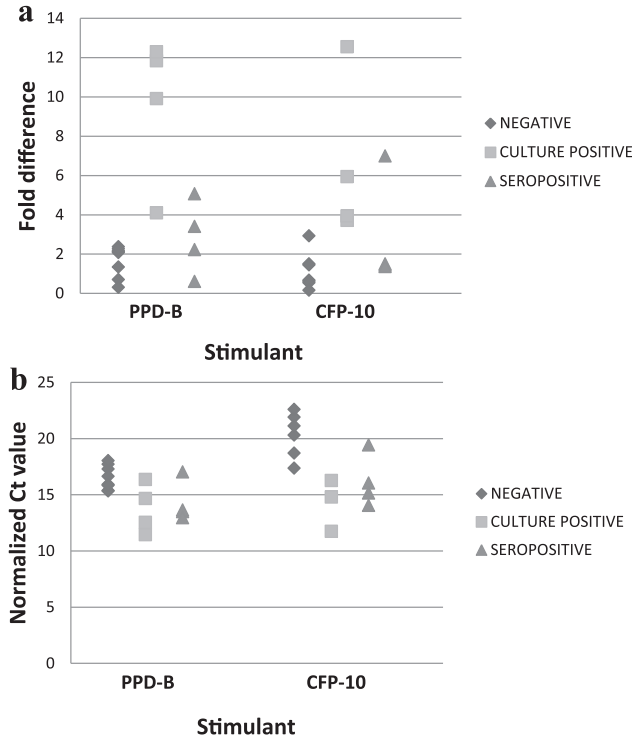

Figure 5. Graphs depict IL-12 (a) and IFN-γ (b) levels in Asian elephant PBMCs following stimulation in culture. Positive samples were from animals with positive trunk wash mycobacterial culture; seropositive animals were culture negative but reactive on both the Elephant TB STAT-PAK® and MAPIA tests. PPD-B = *Mycobacterium bovis* purified protein derivative; CFP-10 = *M. tuberculosis* culture filtrate protein 10.

(Figure 2). These results were in accordance with a previous study comparing baseline cytokine mRNA levels in unstimulated whole blood samples from tuberculosis seropositive and seronegative elephants [15]. Findings of the current study indicate that mitogen/antigen stimulation is effective at inducing TNF-α expression in elephant PBMC cultures, and that samples from tuberculosis positive animals produce higher levels of TNF-α mRNA. In human studies that have evaluated systemic TNF-α levels, both increased and decreased levels have been documented following mycobacterial antigen stimulation of PBMCs from patients as compared with healthy controls [23,27,29,31,41]. With mycobacterial infection, the contribution of TNF-α to local immunity is well-established [42,43]. The significance of this cytokine in the systemic immune response to mycobacterial infection is less well understood. Tumor necrosis factor-α participates in mediation of the systemic inflammatory acute phase response. This response is considered nonspecific and may be initiated by a variety of insults. In addition, a prolonged acute phase response may be detrimental to the host [37].

Elevated systemic TNF-α levels in tuberculosis positive elephants could be a nonspecific component of systemic inflammation associated with infection, or alternatively, the manifestation of a *Mycobacterium* spp. specific prolonged acute phase response. In either case, tuberculosis positive as compared to tuberculosis negative elephants appeared to exhibit exaggerated TNF-α expression in response to mycobacterial antigen stimulation. Lack of differences in TNF-α levels between the groups with ConA stimulation indicated results could not be attributed to nonspecific hyper-reactivity in the positive group. In addition, there was no difference in TNF-α expression between samples from positive or negative elephants in response to nontuberculous mycobacterial antigen (PPD-A) providing evidence that the response noted to CFP-10 and PPD-B was specific to *M. tuberculosis* complex antigens. Though differences in systemic TNF-α levels between positive and negative elephants seem compelling, TNF-α is first and foremost a nonspecific mediator of the innate immune response. Overall health and/or any concurrent disease have the potential to influence TNF-α levels in both positive and negative elephants [15]. Consideration of such information would be essential for accurate interpretation of TNF-α levels for the purpose of tuberculosis diagnosis or treatment monitoring in elephants. In the study animals, no definitive correlation between TNF-α levels and presence of chronic osteoarthritis or sole abscesses could be demonstrated, given the overall low number of unaffected animals ($N = 2$ in the positive group and $N = 4$ in the negative group).

Following both PPD-B and CFP-10 stimulation, levels of IL-12 mRNA were higher in positive samples, and differences in levels between the positive and negative groups were statistically significant (Figure 3). Interleukin-12 is produced by activated macrophages/monocytes and B lymphocytes and results in the differentiation of naïve T lymphocytes into T_H1 cells as well as T_H1 cell production of IFN-γ. Interferon-γ produced by mature T_H1 cells then activates macrophages/monocytes for additional IL-12 production. Asian elephants normally possess high numbers of peripheral blood circulating monocytes [7]. The presence of increased numbers of activated macrophages/monocytes in the positive samples could have led to increased IL-12 expression via an innate or non-*M. tuberculosis* antigen-specific mechanism following stimulation. Examination of direct whole blood smears revealed similar proportions of lymphocytes and monocytes in both positive and negative samples indicating differences between the two groups could not be attributed to discrepancies in absolute cell numbers. Greater expression of IL-12 in samples from tuberculosis positive elephants was also not limited to stimulation with *M. tuberculosis* specific antigens. Stimulation of samples from

positive elephants with PPD-A, a nontuberculous mycobacterial antigen, produced increased IL-12 in comparison with levels in samples from negative elephants. These findings provided evidence for contribution of a non-*M. tuberculosis* antigen-specific response to overall results. However, lack of differences in IL-12 levels between the groups with ConA stimulation indicated above results could not be attributed to nonspecific hyper-reactivity in the positive group. Noted IL-12 expression in samples from positive elephants appeared to be a result of mycobacterial antigen stimulation, although not exclusively specific to *M. tuberculosis* antigens. Altogether findings suggested differences in IL-12 mRNA levels between the positive and negative groups were likely secondary to both antigen-specific and innate/nonspecific responses to stimulation.

Findings of elevated IL-12 mRNA levels in positive samples were contrary to the trend reported in previous human studies where IL-12 levels in samples from healthy controls were higher than levels from patients [30,44–47]. In humans, decreased production of IL-12, a key $T_H1$cytokine, is a manifestation of the inadequate systemic immune response associated with active tuberculosis susceptibility [44–46,48]. Findings in this study indicate tuberculosis positive elephants express IL-12 following stimulation with mycobacterial antigens suggesting aberrations in IL-12 levels alone are not responsible for susceptibility in this species.

In the current study, IFN-γ mRNA could not be detected in the majority of unstimulated sample aliquots. Similar findings were noted in the previous study comparing baseline cytokine mRNA levels in unstimulated whole blood samples from tuberculosis seropositive and seronegative elephants [15]. In the current study, levels of IFN-γ mRNA in most unstimulated sample aliquots were below the threshold of detection despite use of a new assay with enhanced sensitivity. These findings suggested that baseline systemic levels of IFN-γ mRNA in Asian elephants are normally very low. Inability to measure IFN-γ levels in the unstimulated sample aliquots precluded conventional calculation of fold difference using the Pfaffl method. Alternatively, normalized cytokine Ct values were determined for each of the culture stimulants and compared between the two groups. Comparison of IFN-γ normalized, raw Ct values from PPD-B and CFP-10 stimulated sample aliquots revealed significant differences between positive and negative groups. Values of IFN-γ were lower in the positive samples (Figure 4). Because lower normalized Ct values correlate with higher mRNA levels within samples, results indicated increased IFN-γ expression was present in the positive elephant samples.

Many human studies have documented lower IFN-γ levels in stimulated samples from patients as compared with healthy controls [19,30,31,33,49,50]. Fewer human studies have found higher levels of IFN-γ in patients than controls [29,30]. Presumably, this discrepancy among human studies is a consequence of duration of infection at the time of cytokine evaluation. In both susceptible and resistant humans, the initial immune response following infection is characterized by T_H1 domination that includes elevated IFN-γ levels. In the resistant individuals, this response is persistent and prevents active disease. In fact, human IFN-γ-release assays (i.e. QuantiFERON® GOLD) used for the diagnosis of latent tuberculosis rely on detection of this appropriate T_H1 immune response. In susceptible individuals, however, initial robust T_H1 responses dwindle resulting in relative T_H2 dominance and disease progression [21,22,24,51,52]. It is likely that disease stage similarly affects the elephant immune response to tuberculosis.

As an alternative to measuring cytokine protein levels directly, this study relied on detection of cytokine mRNA expression via real time RT-PCR. Protein based detection systems, such as ELISA, are useful for cytokine assessment in humans and many domestic species but require species-specific reagents (antibodies) that are typically not commercially available for most exotic species. As a mRNA based detection system, real time RT-PCR does not require species-specific reagents facilitating its application in any species for which sequence information is available. Because production of many cytokines is primarily transcriptionally regulated, the level of cytokine mRNA present in a sample typically serves as a good estimate of the level of cytokine protein present [53]. Though there is variation among cytokines, previous studies have shown good correlation between cytokine protein levels and mRNA expression in samples [54,55]. Based on this information, cytokine mRNA levels in the elephant samples were presumed to approximate actual protein levels. However, future studies directly measuring cytokine protein levels in elephant samples will be required for confirmation.

In elephants, mycobacterial infection is a chronic, subclinical disease that typically only manifests in aged and/or otherwise stressed individuals [2,3,7]. The nature of the disease in elephants and limitations of current elephant tuberculosis diagnostics impair accurate identification of disease stage in this species [1,8–11]. Consequently, parallels to human tuberculosis immunopathogenesis reliant upon clinical manifestations of disease alone are difficult to extrapolate to elephants. Currently, elephants with positive trunk wash mycobacterial culture are considered to have “active” disease, regardless of presence or absence of clinical signs. Culture negative, seropositive animals have been categorized as “latently” infected by some researchers and clinicians though validity of such a designation with correlation to human latent disease has not been proven. In the current study, division of the positive group into culture positive (“active”) and culture negative, seropositive (“latent”) subgroups revealed distinct and more pronounced trends in IL-12 and, to a lesser extent, IFN-γ levels (Figure 5(a) and (b)). For IL-12, no overlap existed between fold difference values of the four culture positive samples and the negative samples following both PPD-B and CFP-10 stimulation. With PPD-B stimulation, fold difference values for the culture positive samples were among the highest of all samples, and three of the four were 2–3 times higher than any of the culture negative, seropositive samples. For IFN-γ, similar though less pronounced trends were apparent.

Stimulation of elephant PBMCs with mycobacterial antigen did not result in significant expression or suppression of TGF-β, and no difference in TGF-β levels were detected between the positive and negative groups. Results suggest TGF-β may not be an integral component of the elephant systemic immune response to tuberculosis. Alternatively, expression may be disease stage dependent. More potent stimuli, potentially associated with an advanced disease course, may be required to affect production of this cytokine.

Significant differences in proliferative responses between the two groups were apparent following stimulation with both ConA and PPD-B. Stimulation resulted in greater proliferation of positive samples. Though differences in proliferative responses following ConA stimulation suggested some degree of nonspecific, hyper-reactivity may have been present in the positive group, Mann–Whitney *U* analysis of results showed the calculated *U* value for ConA (12) was much higher than the calculated *U* value for PPD-B (0). These results suggested responses following PPD-B stimulation were not due solely to nonspecific hyper-reactivity in the positive samples. Additionally, no significant difference between the groups was noted following PPD-A stimulation suggesting the proliferative response with PPD-B stimulation was specific. In humans with manifestations of advanced tuberculosis-related disease or general immune compromise, evaluation of systemic cytokine levels, specifically IFN-γ, has been shown to have diminished diagnostic sensitivity [56]. In such cases, evaluation of additional parameters may be necessary for reliable diagnosis. Elephant study results indicate lymphocyte proliferative response may be

another valuable parameter to evaluate in cases of confirmed or suspected elephant tuberculosis. Further studies, potentially employing new methodologies (i.e. Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood [FASCIA]) could be useful to better define elephant PBMC proliferative responses with tuberculosis and aid in discrimination between recent infection, active disease, latent disease, and immunological memory of previous exposure [57].

The findings of this study add to the foundation of knowledge on elephant tuberculosis immunopathogenesis. In addition, these results provide validation of PBMC culture techniques for use in evaluation of elephant immune responses. Documentation of differences in functional responses of PBMCs between tuberculosis positive and negative elephants represents the first step towards not only obtaining a better understanding of immunopathogenesis of this disease but also development of new and valuable diagnostic and treatment monitoring options. Measurement of cell-mediated immune responses evidenced by cytokine production of PBMCs following mycobacterial antigen stimulation is the mechanism behind current human first line tuberculosis screening tests (i.e. QuantiFERON® GOLD). These tests are preferred over traditional tuberculin skin tests for screening in humans due to their high-sensitivity and availability of rapid results [58]. A similar test developed for use in elephants could be invaluable for facilitating early diagnosis based on measurement of a cell-mediated immune response, confirming serodiagnostic results, and providing an additional tool for prospective treatment monitoring. Such innovative and directed research will be the key to elucidating elephant tuberculosis susceptibility for the long-term conservation of this charismatic and endangered species.

Acknowledgments

The authors would like to thank the institutions and associated staff that contributed to this work through the donation of samples. The authors would also like to specifically acknowledge Ringling Brothers Center for Elephant Conservation and the Performing Animal Welfare Society. This study was supported by Morris Animal Foundation grant D09ZO-025 and a grant provided by the Chicago Zoological Society/Chicago Board of Trade Endangered Species Fund.

Ethical approval: Not required.

Funding: As stated in the above Acknowledgments section, funding support was provided by Morris Animal Foundation Grant D09ZO-025 and a grant from the Chicago Zoological Society/Chicago Board of Trade Endangered Species Fund.

Competing interests: None declared.

References

[1] Mikota SK, Dumonceaux G, Miller M, Gairhe K, Giri K, Cheeran JV, Abraham D, Lyashchenko K, Larsen RS, Payeur J, Waters WR, Kaufman G. Tuberculosis in elephants: an update on diagnosis and treatment; implications for control in range countries. In: Proceedings: International Elephant Conservation and Research Symposium; 2006. pp. 108–18.
[2] Mikota SK, Maslow JN. Tuberculosis at the human–animal interface: an emerging disease of elephants. Tuberculosis 2011;91:208–11.
[3] Mikota SK, Peddie L, Peddie J, Isaza R, Dunker F, West G, Lindsay W, Larsen RS, Salman MD, Chatterjee D, Payeur J, Whipple D, Thoen C, Davis DS, Sedgwick C, Montali RJ, Ziccardi M, Maslow J. Epidemiology and diagnosis of *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). J Zoo Wildl Med 2001;32:1–16.
[4] Payeur JB, Jarnagin JL, Marquardt JG, Whipple DL. Mycobacterial isolations in captive elephants in the United States. Ann NY Acad Sci 2002;969:256–8.
[5] Angkawanish T, Wajjwalku W, Sirimalaisuwan A, Mahasawangkul S, Kaewsakhorn T, Boonsri K, Rutten VP. *Mycobacterium tuberculosis* infection of domesticated Asian elephants, Thailand. Emerg Infect Dis 2010;16:1949–51.
[6] Verma-Kumar S, Abraham D, Dendukuri N, Cheeran JV, Sukumar R, Balaji KN. Serodiagnosis of tuberculosis in Asian elephants (*Elephas maximus*) in Southern India: a latent class analysis. PLoS One 2012;7:e49548.
[7] Schmitt DL. Proboscidae (elephants). In: Fowler ME, Miller RE, editors. Zoo and wild animal medicine. 5th ed. St. Louis: Saunders; 2003. pp. 545–7.
[8] Lyashchenko KP, Greenwald R, Esfandiari J, Mikota S, Miller M, Moller T, Vogelnest L, Gairhe KP, Robbe-Austerman S, Gai J, Waters WR. Field application of serodiagnostics to identify elephants with tuberculosis prior to case confirmation by culture. Clin Vaccine Immunol 2012;19:1269–75.
[9] Lyashchenko K, Greenwald R, Esfandiari J, Olsen JH, Ball R, Dumonceaux G, Dunker F, Buckley C, Richard M, Murray S, Payeur JB, Anderson P, Pollock JM, Mikota S, Miller M, Sofranko D, Waters WR. Tuberculosis in elephants: antibody responses to defined antigens of *Mycobacterium tuberculosis*, potential for early diagnosis, and monitoring of treatment. Clin Vaccine Immunol 2006;13:722–32.
[10] Greenwald R, Lyashchenko O, Esfandiari J, Miller M, Mikota S, Olsen JH, Ball R, Dumonceaux G, Schmitt D, Moller T, Payeur JB, Harris B, Sofranko D, Waters WR, Lyashchenko KP. Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants. Clin Vaccine Immunol 2009;16:605–12.
[11] Larsen SR, Kay M, Triantis J, Salman MD. Update on serological detection of *Mycobacterium tuberculosis* infection in Asian elephants. In: Proceedings: AAZV, AAWV, AZA/NAG Joint Conference; 2005. pp. 62–3.
[12] Michalak K, Austin C, Diesel S, Bacon JM, Zimmerman P, Maslow JN. *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. Emerg Infect Dis 1998;4:283–7.
[13] Murphree R, Warkentin JV, Dunn JR, Schaffner W, Jones TF. Elephant-to-human transmission of tuberculosis, 2009. Emerg Infect Dis 2011;17:366–71.
[14] De Jong WW, van Dijk MA, Poux C, Kappe G, van Reede T, Madson O. Indels in protein-coding sequences of Euarchontoglires constrain the rooting of the eutherian tree. Mol Phylogenet Evol 2003;28:328–40.
[15] Landolfi JA, Mikota SK, Chosy J, Lyashchenko PD, Giri K, Gairhe K, Terio KA. Characterization of systemic immune responses in Asian elephants (*Elephas maximus*) seropositive for *Mycobacterium* spp. J Zoo Wildl Med 2010;41:445–55.
[16] Landolfi JA, Schultz SA, Mikota SK, Terio KA. Development and validation of cytokine quantitative, real time RT-PCR assays for characterization of Asian elephant immune responses. Vet Immunol Immunopathol 2009;131:73–8.
[17] Sreekumar E, Janki MBV, Arathy DS, Hariharan R, Avinash D, Premraj C, Rasool TJ. Molecular characterization and expression of interferon-γ of Asian elephant (*Elephas maximus*). Vet Immunol Immunopathol 2007;118:75–83.
[18] Bhatt K, Salgame P. Host innate immune response to *Mycobacterium tuberculosis*. J Clin Immunol 2007;27:347–62.
[19] Bhattacharyya S, Singla R, Dey AB, Prasad HK. Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis. Infect Immun 1999;67:5597–603.
[20] Boom WH, Canaday DH, Fulton SA, Gehring AJ, Rojas RE, Torres M. Human immunity to *M. tuberculosis*: T cell subsets and antigen processing. Tuberculosis 2003;83:98–106.
[21] Flynn JL, Chan J. Immunology of tuberculosis. Annu Rev Immunol 2001;19:93–129.
[22] Kaufmann SH, Cole ST, Mizrah V, Rubin E, Nathan C. *Mycobacterium tuberculosis* and the host response. J Exp Med 2005;201:1693–7.
[23] Kellar KL, Gehrke J, Weis SE, Mahmutovic-Mayhew A, Davila B, Zajdowicz MJ, Scarborough R, LoBue PA, Lardizabal AA, Daley CL, Reves RR, Bernardo J, Campbell BH, Whitworth WC, Mazurek GH. Multiple cytokines are released when blood from patients with tuberculosis is stimulated with *Mycobacterium tuberculosis* antigens. PLoS One 2011;6:e26545.
[24] Raja A. Immunology of tuberculosis. Indian J Med Res 2003;120:213–32.
[25] Rhodes SG, Buddle BM, Hewinson RG, Vordermeier HM. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology 2000;99:195–202.
[26] Thacker TC, Palmer MV, Waters WR. Correlation of cytokine gene expression with pathology in white-tailed deer (*Odocoileus virginianus*) infected with *Mycobacterium bovis*. Clin Vaccine Immunol 2006;13:640–7.
[27] Wang X, Jiang J, Cao Z, Yang B, Zhang J, Cheng X. Diagnostic performance of multiplex cytokine and chemokine assay for tuberculosis. Tuberculosis 2012;92:513–20.
[28] Welsh MD, Cunningham RT, Corbett DM, Girvin RM, McNair J, Skuce RA, Bryson DG, Pollock JM. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology 2005;114:101–11.
[29] Al-Attiyah R, Madi NM, El-Shamy AM, Wiker HG, Anderson P, Mustafa AS. Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis*. FEMS Immunol Med Microbiol 2006;47:254–61.
[30] Demissie A, Abebe M, Aseffa A, Rook G, Fletcher H, Zumla A, Weldingh M, Brock I, Anderson P, Doherty TM. Healthy individuals that control a latent infection with *Mycobacterium tuberculosis* express high levels of Th1 cytokines and the IL-4 antagonist IL-4δ2. J Immunol 2004;172:6938–43.
[31] Hussain R, Kaleem A, Shahid F, Dojki M, Jamil B, Mehmood H, Dawood G, Dockrell HM. Cytokine profiles using whole-blood assays can discriminate between tuberculosis patients and healthy endemic controls in a BCG-vaccinated population. J Immunol Methods 2002;264:95–108.

[32] Surcel HM, Troye-Blomberg S, Paulie S, Andersson G, Moreno C, Pasvol G, Ivanyi J. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 1994;81:171–6.
[33] Torres M, Herrara T, Villareal H, Rich EA, Sada E. Cytokine profiles for peripheral blood lymphocytes from patients with active pulmonary tuberculosis and healthy household contacts in response to the 30-kilodalton antigen of *Mycobacterium tuberculosis*. Infect Immun 1998;66:176–80.
[34] Walzi G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. Nat Rev Immunol 2011;11:343–54.
[35] Landolfi JA. Immune function alterations in Asian elephants (*Elephas maximus*) infected with Mycobacterium species [Dissertation]. Urbana-Champaign: University of Illinois; 2013., http://hdl.handle.net/2142/45371.
[36] Pfaffl MW. A new mathematical model for relative quantification in real time RT-PCR. Nuc Acids Res 2001;29:2002–7.
[37] Abbas AK, Lichtman AH. Effector mechanisms of immune responses. In: Abbas AK, Lichtman AH, editors. Cellular and molecular immunity. 5th ed. Philadelphia: Saunders; 2003. pp. 241–345.
[38] Garnier TK, Eiglmeir K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, Simon S, Harris B, Atkins R, Doggett J, Mayes R, Keating L, Wheeler PR, Parkhill J, Barrell BG, Cole ST, Gordon SV, Hewinson RG. The complete genome sequence of *Mycobacterium bovis*. Proc Natl Acad Sci USA 2003;100:7877–82.
[39] Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, Shigeto E, Harada N, Mitarai S, Okada M, Suzuki K, Inoue Y, Tsuyuguchi K, Sasaki K, Mazurek GH, Tsuyuguchi I. Specific detection of tuberculosis infection an interferon-γ-based assay using new antigens. Am J Respir Crit Care Med 2004;170:59–64.
[40] Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, Hewinson RG. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 2001;8:571–8.
[41] Kim SY, Park MS, Kim YS, Kim SK, Chang J, Lee HJ, Cho SN, Kang YA. The responses of multiple cytokines following incubation of whole blood from TB patients, latently infected individuals and controls with the TB antigens ESAT-6, CFP-10 and TB7.7. Scand J Immunol 2012;76:580–6.
[42] Cavalcanti YV, Brelaz MC, Neves JK, Ferraz JC, Pereira VR. Role of TNF-alpha, IFN-gamma, and IL-10 in the development of pulmonary tuberculosis. Pulm Med 2012;2012:745483.
[43] Ehlers S. Role of tumor necrosis factor (TNF) in host defense against tuberculosis: implications for immunotherapies targeting TNF. Ann Rheum Dis 2003;62(Suppl. II):ii37–42.
[44] Cooper AM. Cell-mediated immune responses in tuberculosis. Annu Rev Immunol 2009;27:393–422.
[45] Cooper AM, Solache A, Khader SA. Interleukin-12 and tuberculosis: an old story revisited. Curr Opin Immunol 2007;19:441–7.
[46] Méndez-Samperio P. Role of interleukin-12 family cytokines in the cellular response to mycobacterial disease. Int J Infect Dis 2010;14:e366–71.
[47] Song C, Kim H, Park J, Lim J, Kim U, Kim J, Paik T, Kim K, Suhr J, Jo E. Depressed interleukin-12 (IL-12), but not IL-18, production in response to a 30- or 32-kilodalton mycobacterial antigen in patients with active pulmonary tuberculosis. Infect Immun 2000;68:4477–84.
[48] Azad AK, Sadee W, Schlesinger LS. Innate immune gene polymorphisms in tuberculosis. Infect Immun 2012;80:3343–59.
[49] Sanchez FO, Rodriguez JI, Agudelo G, Garcia LF. Immune responsiveness and lymphokine production in patients with tuberculosis and healthy controls. Infect Immun 1994;62:5673–8.
[50] Seah GT, Rook GAW. High levels of mRNA encoding IL-4 in unstimulated peripheral blood mononuclear cells from tuberculosis patients revealed by quantitative nested reverse transcriptase-polymerase chain reaction; correlations with serum IgE levels. Scand J Infect Dis 2001;33:106–9.
[51] Boussiotis VA, Tsai EY, Yunis EJ, Thim S, Delgado JC, Dascher C, Berezovskaya A, Rousset D, Reynes J, Goldfeld AE. IL-10-producing T cells suppress immune responses in anergic tuberculosis patients. J Clin Invest 2000;105:1317–24.
[52] Dlugovitzky D, Torres-Morales A, Rateni L, Farroni MA, Largacha C, Molteni O, Bottasso O. Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. FEMS Immunol Med Microbiol 1997;18:203–7.
[53] Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci USA 1990;87:2725–9.
[54] Doherty TM, Demissie A, Menzies D, Andersen P, Rook G. Effect of sample handling on analysis of cytokine responses to *Mycobacterium tuberculosis* in clinical samples using ELISA, ELISPOT and quantitative PCR. J Immunol Methods 2005;298:129–41.
[55] Shebl FM, Pinto LA, García-Piñeres A, Lempicki R, Williams M, Harro C, Hildesheim A. Comparison of mRNA and protein measures of cytokines following vaccination with human papillomavirus-16 L1 virus-like particles. Cancer Epidemiol Biomarkers Prev 2010;19(4):978–81.
[56] Lee J, Lee SY, Won DI, Cha SI, Park JY, Kim CH. Comparison of whole-blood interferon-γ assay and flow cytometry for the detection of tuberculosis infection. J Infect 2013;66:338–45. http://dx.doi.org/10.1016/j.jinf.2012.08.020.
[57] Borgstrom E, Andersen P, Andersson L, Julander I, Kallenius G, Maeurer M, Norrby M, Rosenkrands I, Tecleab T, Bruchfeld J, Gaines H. Detection of proliferative responses to ESAT-6 and CFP-10 by FASCIA assay for diagnosis of *Mycobacterium tuberculosis* infection. J Immunol Methods 2011;370:55–64.
[58] Pai M, Zwerling A, Menzies D. Systemic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. Ann Intern Med 2008;149:177–84.